STUDIES IN THE PHYSIOLOGY OF THE FUNGI

IX. Enzyme Action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr.

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Careful studies in the physiology of the wood-destroying fungi have but recently received the degree of consideration to which their economic importance entitles them. Many economic forms are as yet untouched, and it is our purpose to study some of the fundamental physiological relations existing between fungus and host. The following is the first of a series of investigations concerning especially the enzyme activities of such forms. It is recognized that *Armillaria mellea* has received considerable attention in respect to its physiological relations because of its importance as a root rot of fruit trees, but, as far as the writers are aware, there has been no physiological study of *Daedalea confragosa* or *Polyporus lucidus*, both of which must be recognized as important wood-rotting fungi.

In a recent paper by one of us[1] the literature and methods of enzyme study in the wood-destroying fungi have been sufficiently reviewed, so that in the present paper only specific references to previous literature will be made, and unless otherwise stated, the methods followed will be those previously described.

The fungi from which was obtained the fungous meal used in the present study were grown on sterile, sliced carrot in large Erlenmeyer flasks. While still in an active growing condition the fungous mats were removed and rapidly air-dried by means

[1] Zeller, S. M. Studies in the physiology of the fungi. II. Lenzites saepiaria Fries, with special reference to enzyme activity. Ann. Mo. Bot. Gard. 3: 439–512. *pl. 8–9.* 1916.

of an electric fan. When thoroughly dry the material was finely ground.

ESTERASES

In the study of the esterases of these three fungi methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion were used as substrates. When esterases act upon esters fatty acids are liberated, and thus the concentration of the active acidity can be used as an index of the degree of enzyme action. First, a determination was made of the hydrogen ion concentration of the substrate. A similar determination was also made of the substrate to which a certain amount of autoclaved fungous meal had been added. These two determinations did not always check, due to the introduction of certain substances with the fungous meal and perhaps also to certain buffer effects. The latter determination was taken as the control in each case and compared with a third determination made of the substrate to which a similar amount of fungous meal had been added and incubated twenty-one days.

It was found that there was no apparent esterase activity of any of the fungi on any of the substrates except methyl acetate upon which a slight esterase activity was shown in the case of *Daedalea confragosa* and *Polyporus lucidus*. These results are similar to those found for *Lenzites saepiaria*.[1]

CARBOHYDRASES

The action of carbohydrases was determined upon maltose, lactose, sucrose, raffinose, potato starch, inulin, cellulose from various sources, and hemicellulose. The amount of sugars which reduce Fehling's solution in the enzyme cultures after incubation was taken as the index of enzyme activity. Since this study is merely to indicate the relative activity between the different fungi on the different substrates the results are given as the number of cc. of N/20 potassium permanganate required to oxidize the dissolved copper oxide. The results in the following table are the averages of duplicate enzyme cultures after the Fehling's control had been deducted.

[1] Zeller, S. M., *l. c.*

TABLE I

SHOWING THE ACTION OF CARBOHYDRASES ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

Incubation period	Substrate	P. lucidus			A. mellea		D. confragosa	
		With fungous meal	With fungous meal autoclaved	Without fungous meal	With fungous meal	With fungous meal autoclaved	With fungous meal	With fungous meal autoclaved
		Number of cc. of $\frac{N}{20}$ $KMnO_4$						
14 days	Maltose	36.1	16.3	13.1	36.2	16.3	23.5	14.7
24 days	Lactose	31.7	23.0	18.8	36.9	26.0	24.9	19.3
6 hours	Sucrose	27.5	1.6	1.0	31.3	2.3	11.1	0.3
2 days	Raffinose	32.5	5.2	0.8	12.5	2.6	12.5	0.6
6 hours	Potato starch	19.2	6.1	1.4	25.3	2.6	7.7	1.5
2 days	Inulin	20.3	4.9	0.8	15.4	2.4	7.8	0.9
28 days	Ash cellulose	5.6	0.7	0.3	4.1	0.5	4.3	1.8
28 days	Fir cellulose	5.8	0.3	0.2	3.3	0.4	4.7	0.4
28 days	Oak cellulose	3.8	0.2	0.1	3.6	0.1	3.1	1.3
28 days	Hemicellulose	4.4	0.3	0.1	2.9	0.1	3.2	1.8

In general the carbohydrate activity is greater in *Polyporus lucidus* and *Armillaria mellea* than in *Daedalea confragosa*, with the possible exception of raffinase where the activity in *Daedalea confragosa* approximates that of *Armillaria mellea*. The striking feature of the results is the evident presence of lactase in the three fungi. This is the first record of the presence of lactase in the higher fungi.

In the study of cellulase pure cellulose was prepared from Douglas fir, ash, and red oak, according to the method frequently reported from this laboratory. Suspensions of these in doubly

distilled water were used as substrates. After the enzyme culture had been incubated for 28 days there was a marked increase in the amounts of reducing sugars produced by all the fungi on all of the substrates.

For a hemicellulose substrate cleaned autoclaved endosperms from date seeds were used. These were shaved into very thin slices and placed in distilled water with the fungous meal. Hydrolysis quite comparable to that produced in the cellulose experiments resulted in each case as is indicated in table 1.

EMULSIN

The presence or absence of emulsin was determined by the effect of the fungous meal upon amygdalin, which upon hydrolysis produces glucose, benzaldehyde, and hydrocyanic acid. After incubation of seven days the amount of glucose present in the cultures was determined as in the cases where carbohydrates were used as substrates, and the results are tabulated in the following table:

TABLE II

SHOWING THE ACTION OF EMULSIN ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

Enzyme culture	P. lucidus	A. mellea	D. confragosa
	Number of cc. of $\frac{N}{20}$ $KMnO_4$		
1% amygdalin+ fungous meal	26.0	12.5	18.1
1% amygdalin+ fungous meal (autoclaved)	4.8	0.6	0.1
1% amygdalin	0.6	0.6	0.6

In all cases where the above sugar tests showed evidence of the breaking down of amygdalin the odor of benzaldehyde was easily recognized. There was evidence of emulsin in all three of the fungi used.

TANNASE

In order to determine the tannase activity the gallic acid, which is a product of hydrolysis of tannic acid, was titrated with

standard iodine solution. The results show the presence of tannase in *Polyporus lucidus* and *Daedalea confragosa* but none was detected in *Armillaria mellea*. This fact seems peculiar, since the rhizomorphs of *Armillaria mellea* are usually found next to the inner bark of the woody tissues where the tannin is usually present in the greatest amount for the specific host. The fact that the fungi from which the fungous meal was made were grown on carrot may have had some influence on the production of tannase in this particular instance.

Amidase and Urease

The presence of the enzymes which split amino acids and urea into ammonia and hydroxy acids was demonstrated by using such substrates as asparagin, acetamid, and urea. The usual Folin method of determining the presence of ammonia is such a time-consuming procedure that a new method was devised involving the indicator method of determining the hydrogen ion concentration of solutions. In brief, the method employed is as follows:

The substrate and fungous meal in the desired proportions were placed in wash bottles, the inlets and outlets of which were sealed with rubber tubes and clamps in order to retain any ammonia which might have been given off during the period of incubation. After a period of incubation of seven days the ammonia was drawn directly through another small wash bottle by means of a Richards pump. The small wash bottle contained 10 cc. of doubly distilled water to which was added 6 drops of brom thymol blue made up in the proportions suggested by Clark and Lubs.[1] This doubly distilled water had a hydrogen ion concentration of P_H 5.6, at which concentration the indicator was yellowish brown. Due to the hydrogen ion concentration decreasing as the ammonia is drawn through, the color changes from green to blue. The length of time taken to change from P_H 5.6 to P_H 7.0 would, of course, depend upon the amount of ammonia present, and this was thus taken as a criterion of the

[1] Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact. 2: 1–34, 109–136, 191, 236. 1917.

relative rate of ammonia production in the various enzyme cultures. In no case was the gas drawn through the wash bottle for a period longer than three minutes. At the end of this period the actual hydrogen ion concentration of the distilled water was determined. Sometimes the change was so rapid that it was not necessary to run the experiment for three minutes. In such instances other indicators having a wider alkaline range were substituted for brom thymol blue. In the urea control there was a change from P_H 5.6 to P_H 6.0, and thus, for urea, changes not going beyond P_H 6.0 were considered as negative. The results are tabulated in table III.

Urease was demonstrated for the three fungi. It was most pronounced in *Daedalea confragosa* and least in *Polyporus lucidus*. Only *Armillaria mellea* showed slight amidase action when acetamid was used as a substrate. There was no amidase action when asparagin was used as a substrate.

Due to the fact that traces of alkalis or acids cause considerable shifting of the hydrogen ion concentration in such an unbuffered solution as doubly distilled water it is believed that this method can be used to determine the presence of minute traces of ammonia which would be undetectable by the methods usually employed, and the determination is much more rapid. In the present paper only relative determinations were necessary but there is no valid reason why quantitative determinations could not be made by this method.

PROTEASES

Tryptic and ereptic fermentation was studied by the use of albumin, peptone, casein, legumin, and fibrin in enzyme cultures having a neutral, acid, and alkaline reaction. When fibrin was used as a substrate positive results were obtained to show the presence of both trypsin and erepsin in all three of the fungi. These results were most pronounced in the cultures having an acid reaction and least in those with an alkaline reaction. In the case of the plant protein, legumin, there was very slight indication of the presence of tryptic and ereptic fermentation only in *Polyporus lucidus* and only when the substrate was acid in reaction. In *Polyporus lucidus* there was indication of the

presence of trypsin when albumin of acid reaction was used. This was not true of *Armillaria mellea* or *Daedalea confragosa*. In no case was there a splitting of peptone or casein.

TABLE III

UREASE AND AMIDASE ACTIVITY IN ARMILLARIA MELLEA, DAEDALEA CONFRAGOSA, AND POLYPORUS LUCIDUS

	Enzyme culture	No.	Change in H ion concentration. P_H values		
			Urea	Acetamid	Asparagin
P. lucidus	Substrate + fungous meal	1	5.6–6.8 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–6.6 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–5.8 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
A. mellea	Substrate + fungous meal	1	5.6–7.2 30 sec.	5.6–6.0 3 min.	5.6–5.6 3 min.
		2	5.6–7.2 25 sec.	5.6–6.0 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
D. confragosa	Substrate + fungous meal	1	5.6–7.2 1 sec.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–8.8 3 sec.	5.6–5.6 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
Control	Substrate alone	1	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.

SUMMARY

In *Polyporus lucidus* the presence of the following enzymes is demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

In *Armillaria mellea* the presence of the following enzymes is demonstrated: maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, urease, amidase, and trypsin and erepsin when fibrin is used as a substrate.

In *Daedalea confragosa* the following enzymes are present: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

A new method for the determination of ammonia liberated by amidase is described. This method involves the application of the indicator method for hydrogen ion concentration determination.

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